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Final Technical Report, ONR Code 342 (Biology)

Cover Page:

GRANT # : N000140910215

PRINCIPAL INVESTIGATOR : Philip LeDuc

INSTITUTION: Carnegie Mellon University

GRANT TITLE: Investigating Cell-Material Interactions of *Magnetospirillum magneticum* as an Approach for Probing Submerged Surface Structural Integrity

AWARD PERIOD: January 1, 2009-July 1, 2012

20130213284

OBJECTIVE: To investigate the relationship between magnetic field gradients and cell-material interactions using integrated experimental and computational approaches in a multidisciplinary effort; to develop an experimental system to create magnetized structures for examining cell interactions; to examine the magnetic response of surfaces through a computational analysis approach; to probe the response of *Magnetospirillum magneticum* to localized magnetic fields with cell-material interactions; to understand the genetic response of these magnetotactic bacteria to applied magnetic fields.

APPROACH: Our overall approach was to build a combined experimental and computational approach for investigating *Magnetospirillum magneticum* in cell-material interactions with respect to magnetic field gradients. We believe that this may lead to new non-destructive evaluation techniques for examining the structural integrity of naval structures, such as ship hulls, with predictive capabilities. We have accomplished this by investigating the magnetization of structures like metal plates with defects (Specific Objective 1), and then modeling them to predict their magnetic field gradients (Specific Objective 2), and investigating the response of the bacteria to the magnetic fields (Specific Objective 3). We also have examined their genetic responses under magnetic field stimulation.



Figure 1. Offset Helmholtz Coil system, microscope and custom-designed microcontroller

ACCOMPLISHMENTS:

Summary of Accomplishments

We have developed a microscope-based, offset Helmholtz coil system with a custom-designed microcontroller. We have developed a microfabrication approach for sharper and “soft” magnetic materials. We have built finite element approaches for understanding localized magnetic field responses. We have implemented an experimental model system using ferromagnetic beads. We have applied direct and frequency based magnetic fields for controlling magnetotactic bacteria (MTB) movement. We have created chained patterns of living MTB for deployment of spatially organized MTB populations and examined individual MTB motion. We have continued working toward developing a gene expression system in MTB. We have examined the genetic response of MTB to magnetic fields.

Expanded Accomplishments

We have developed a microscope-based, offset Helmholtz coil system with a custom-designed microcontroller. To be able to determine the speed and positioning of the MTB as magnetic fields were applied, we built both a large and small Helmholtz Coil pair with differential field strengths (Fig. 1). Each coil in the set of the small Helmholtz Coils was 5” in diameter and had 400 turns. The large set of coils was 23” in diameter and each had 150 turns. The smaller Helmholtz coils showed greater field strength per given current than the large coils pair. We creatively combined the 2 sets of Helmholtz coils to form an offset Helmholtz coils

system. We used this approach to alternate magnetic field directions that we were able to impose on the bacteria. We also could control the angle by which the pair of coils is offset (see Figure 1). We then programmed an Arduino Uno microcontroller to rapidly alternate between the two coils. A circuit with a transistor (TIP102) and diode (1N4004) was built to protect the Arduino as the maximum current for the Arduino is about 30 mA and we needed over 0.1 A to drive the coils. In the circuitry, we included LED lights to indicate which coils were enabled at controlled times and allow registration of this information.

We developed a microfabrication approach for sharper and “soft” magnetic materials

Our advanced fabricated structure with sharp features and 30 nm thicknesses were needed for a number of reasons including as magnetic field concentrators (Fig. 2). Ferromagnetic materials are categorized into “soft” and “hard” magnetic materials. “Soft” magnetic materials are easier to demagnetize and “hard” magnetic materials (permanent magnets) are harder to demagnetize. To demagnetize a ferromagnetic material a threshold based on the coercivity (H_c) has to be reached. We implemented Permalloy (NiFe), which is a soft magnetic material, because the field produced by our system (Helmholtz Coils) was strong enough to magnetize these structures in a preferred orientation due to its high permeability $\mu = B/H$. Also the magnetic remanence of Permalloy was low enough that when we removed the external magnetic field, we could reuse these structures, which was important for various reasons including cost, repeatability, etc. A chrome quartz mask with a tolerance of $0.5\mu\text{m}$ allowed us to fabricate sharp features suited for the concentration of magnetic fields. Earlier in the project, our resolution was limited to $10\mu\text{m}$ along with having lift-off issues.

We developed finite element approaches for understanding localized magnetic field responses.

Using a Finite Element Methods magnetics (FEMM) approach, we developed a 2D planar axisymmetric simulation of the Helmholtz Coils with the same parameters of the physical

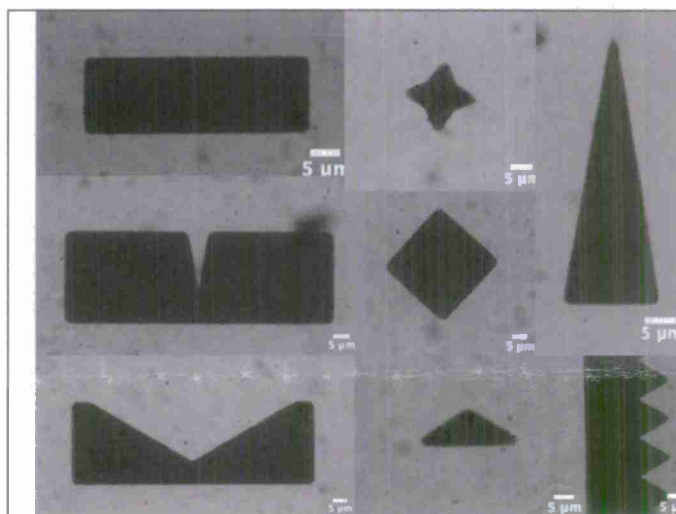


Figure 2. Microfabricated Permalloy islands with controlled shapes

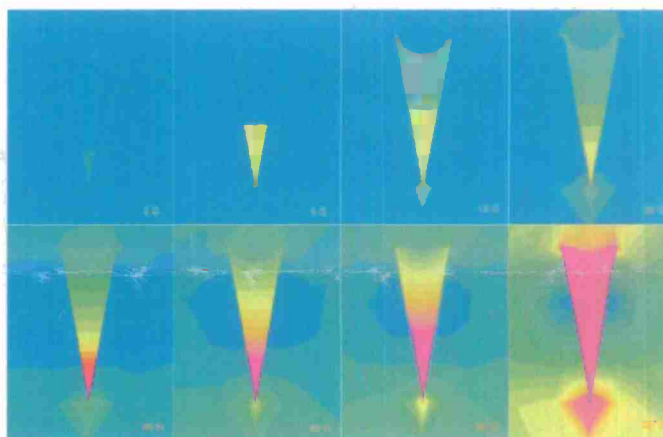


Figure 3. Simulations of magnetic field concentration as the field is increased until a saturation level is reached.

system previously described. The simulation results showed that the sharp magnetic features in the structure concentrated the field lines (Fig. 3). The magnetic flux density was higher at specific protrusions and corners. Increasing the magnetic field strength though did not necessarily correlate to higher field concentrations at these sharp points of the microfabricated islands. A point of saturation existed where further increases in magnetic field strength did not improve the flux density (Fig. 3). We developed a simulation to analyze the magnetic intensity on the islands as we increased the magnetic field from 1G to 227G. At approximately 227G the islands saturated and the magnetic field spread across the islands.

We implemented an experimental model system using ferromagnetic beads

To examine the existence of a local magnetic gradient and to experimentally quantify the field near the islands, we used ferromagnetic beads as a model system. We implemented the differential equation in equation (1) and solved for F_{mag} . This differential equation balanced the

$$\rho V \hat{a} = F_{mag} - F_s \quad (1)$$

$$F_s = 6\pi\eta Rv \quad (2)$$

magnetic and drag forces (Stokes's law) shown in equation (2) where η was the viscosity, R was the radius of the spheres and v was the terminal velocity. Using this approach, the magnitude of the force that the island exerted on the beads based upon distance from the edge of the square island was determined (Fig. 4). At a low magnetic field the island established a magnetic field gradient that imposed a force attracting the beads toward the island if they were close. However, when the magnetic field was higher the beads were attracted with the magnetic gradient at over twice the distance. Figure 5 shows the accumulation of the magnetic beads at the sharp features of the triangle and square islands, which correspond to the regions where the magnetic field lines were concentrated.

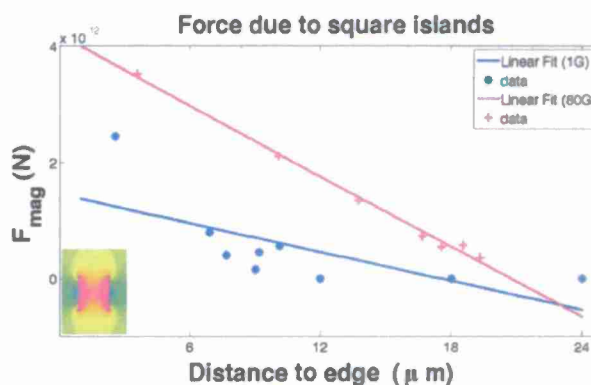


Figure 4. Force versus distance to the square island at 1G and 80G magnetic field strengths.

We applied direct and frequency based magnetic fields for controlling magnetotactic bacteria movement.

We used the offset Helmholtz coils system previously described to controllably change the magnetic field direction rapidly to study the response of the magnetic beads as a model system and then applied this approach to our magnetotactic bacteria. The combination of the velocity vector of the bacteria when it was moving and the speed of the deflection of the bacteria from its flagella induced direction dictated the need to determine how quickly the bacteria responded to external magnetic fields. We used a frequency based external magnetic field application and an associated analysis of the dynamic response of the beads and the bacteria to infer their response.

We have created chained patterns of living MTB for deployment of spatially organized MTB populations.

Current state-of-the-art magnetic particle inspection (MPI) methods probe structural integrity of metal objects using chains of beads instead of individual beads to get a better indication of the presence of a crack or defect. We believe that using a similar approach through forming a long chain of individual magnetotactic bacteria so that they can bridge across a crack will have significant advantages. For example, the position of this chain can be used to determine defects in metallic structures. To attach the magnetotactic bacteria together to form these chains, we used biotin-streptavidin to link adjacent magnetotactic bacteria. We made this link through proteins of the flagella of the bacteria and enabled end-to-end binding. We used anti-FLIC flagellin antibody that recognizes FliC. Via a TRITC molecule conjugated to this flagellin antibody, we allowed the flagellin antibody-TRITC conjugation system to attach to the magnetotactic bacteria flagella and then observed under a magnetic field the fluorescence of the magnetic bacteria moving along the field lines. We then created a chain of magnetotactic bacteria. We grew one population of MTBs with the flagellin antibody conjugated to biotin and another population of MTBs with the flagellin antibody conjugated to streptavidin; we then cultured them together to form the chains.

Having magnetic structures with defined shapes allowed the re-direction of the MTBs as they approached concentrated magnetic field line regions. Magnetic field lines were altered when they approached magnetic materials and thus magnetotactic bacteria would deflect indicating the shape of the magnetic field lines. For instance, in a sharp/deep side-notch magnetic island (Fig. 6), the MTB were analyzed both before they approached the notch and close to the notch thus examining the effects on the path of the bacteria that the local concentrated magnetic fields would have on the bacteria movement. As MTB moved from the left side of the image to the right side (Fig. 6), the bacteria

began in the horizontal direction while away from the notch and then as the bacteria approached the notch, they deflected down toward the notch due to its magnetic concentration gradient.

Furthermore, using a unique four-point star concentrator (Fig. 7), we found MTB under magnetic stimulation had three repeatable, distinct behaviors when MTB moved near local fields

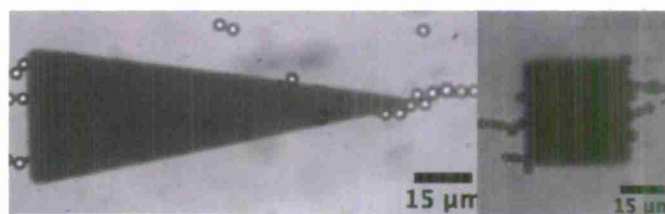


Figure 5. Magnetic beads accumulation at the magnetic field concentrated regions of the islands.

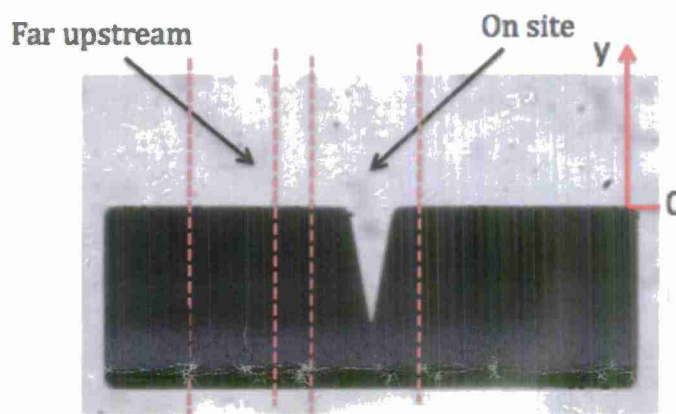


Figure 6. MTB moving near a side notched island with magnetic fields applied. The dotted lines delineate regions that were examined in terms of bacteria orientation. These are close to and away from the notch in the island.

(gradient fields) created by these concentrating microstructures: continuous, reverse and double reverse movements (Fig. 7). The continuous bacteria response was characterized by bacteria swimming close to the Permalloy island without displaying any abrupt reverse of direction. Alternatively, in the "reverse" case, the bacterium initially swam in one direction as it approached a concentrator, and then completely reversed directions. In the double reverse case,

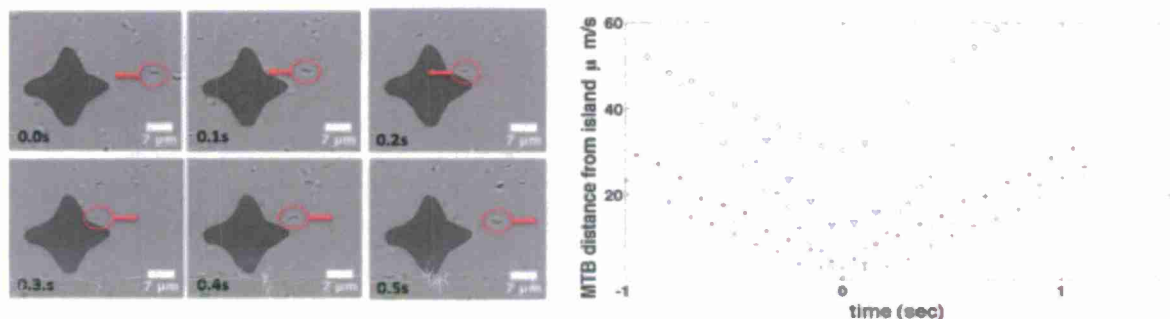


Figure 7. Reversal motion of magnetotactic bacteria as they approached magnetic field concentrators.

the bacteria performed two reverse motions, and thus, after the two changes of direction, they swam away from the concentrator in the same (original) direction as they approached the concentrator. This reversing response was also directly related to the speed of the MTB. Only bacteria swimming at lower speeds had this reversal behavior.

We have continued working toward developing a gene expression system in MTB.

A pBBR1Km-GFP vector was constructed by ligation of the GFP coding sequence into dual-origin, broad-host range plasmid pBBR1MCS-2. This vector contained the ColE1 origin of replication for high-copy expression in *E. coli*, useful for harvesting large amounts of DNA, as well as the pBBR1 replicon, which conferred the vector the ability to replicate in many gram-negative bacterial hosts. Electroporation was not found to be a successful method of introducing the shuttle vector into *Magnetospirillum magneticum* AMB-1. To separate electroporation lethality from ineffective replication of the shuttle vector, the function of the pBBR1 replicon in the constructed vector pBBR1Km-GFP was tested in the hosts:

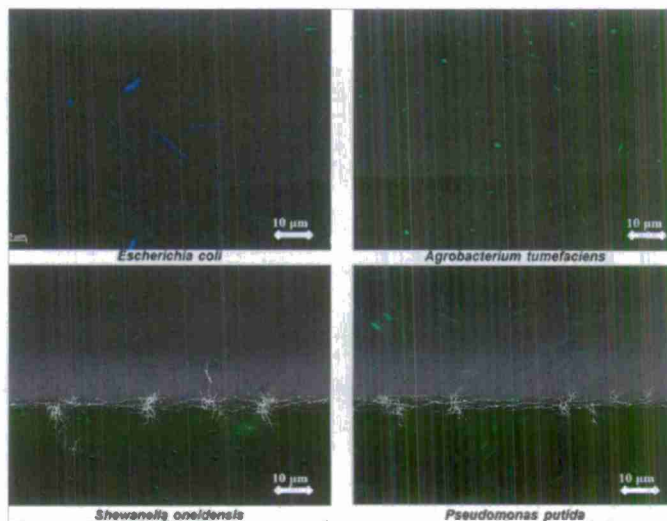


Figure 8. GFP fluorescence of gram-negative hosts with our plasmid pBBR1Km-GFP

Agrobacterium tumefaciens LBA4404, *Shewanella oneidensis* MR-1, and *Pseudomonas putida* KT2440. Replication and expression of the vector was positively confirmed by observation of GFP fluorescence of transformants (Fig. 8). Thus, transconjugation was selected to introduce the shuttle vector into *M. magneticum* AMB-1 rather than electrotransformation. The vector was transformed into the *E. coli* strain S17-1, which provides chromasomally-integrated RK2 mobilization functions necessary to mediate conjugative plasmid transfer. This donor strain was then conjugally mated to *M. magneticum* recipients, yielding MTB transconjugants with conferred antibiotic resistance, but with a very low fraction of cells indicating GFP fluorescence.

A diverse range of variables was tested including those published in the limited papers in the literature showing successful expression. Even though this project's funding cycle is completed, we continue to work on this today.

We have examined the genetic response to magnetic fields.

We have worked with AMB-1 in examining their genetic response to applied magnetic fields focusing on likely candidates in the magnetosome island (MAI), magnetotactic islet, flagellar, and cytoskeleton genes. These genes were chosen because we expect that increasing ambient field will require cells to exert a greater force to turn

and they may attempt to adapt to the atypical stimulus by altering gene expression related to magnetosomes themselves, cytoskeletal elements that provide internal magnetosome scaffolding, or motility genes. After extracting total RNA and conversion to cDNA, Quantitative Real Time-PCR (qRT-PCR) was performed to measure relative transcriptional levels using the ABI Prism 7000 Sequence Detection System. Specific oligonucleotide primer sets were designed and validated to detect MAI genes (*mps*, *magA*, *mamE*, *mamK*, *mms6*, *mms7*, *mms13*, *mms16* and *mms24*); magnetotactic islet genes (*mamE*-like and *mamK*-like); flagellar genes (*flagellinA*, *flagellinB*, *fliF*, *fliG* and *fliE*); cytoskeletal genes (*mreB* and *mreC*); and a housekeeping gene, a 16S ribosomal subunit. For magnetic stimulation experiments, cycle threshold (C_T) values for transcription levels were obtained and normalized to 16S to determine the ΔC_T value. ΔC_T values for magnetic field treatment groups were compared to controls using the $\Delta\Delta C_T$ method to give relative fold-change in gene expression.

We tested the effect of high magnetic fields (10G) on AMB-1 gene expression. After equilibrating a solenoid within a 27°C incubator and subsequently monitoring the temperature at the center of the solenoid throughout the experiment, tubes of AMB-1 cells in liquid culture were placed at the center of the solenoid and kept under a constant 10G field for 1 hour, 3 hours or 8 hours. All culture tubes were grown under microaerobic conditions as specified by ATTC® protocols (i.e., no gas headspace in culture vessels) and began the experiment at early-log phase. At each time point, three tubes of magnetically-stimulated AMB-1 cells and three tubes of non-stimulated (control) cells were harvested for total RNA, which was subsequently converted to cDNA for qRT-PCR analysis. Transcription was immediately stabilized with Qiagen®'s RNA Protect reagent. In addition, a group of cells were exposed to ambient air for 8 hours to determine the effect of oxygen on AMB-1 gene expression. Both high magnetic field and oxygen exposure altered gene expression at the time points tested (Fig. 9).

After 1 hour of magnetic stimulation, gene expression was drastically down-regulated for all genes tested; whereas, gene expression was slightly up-regulated after 3 hours. This suggests that magnetic field stimulation may lead to altered signaling within AMB-1 related to MAI

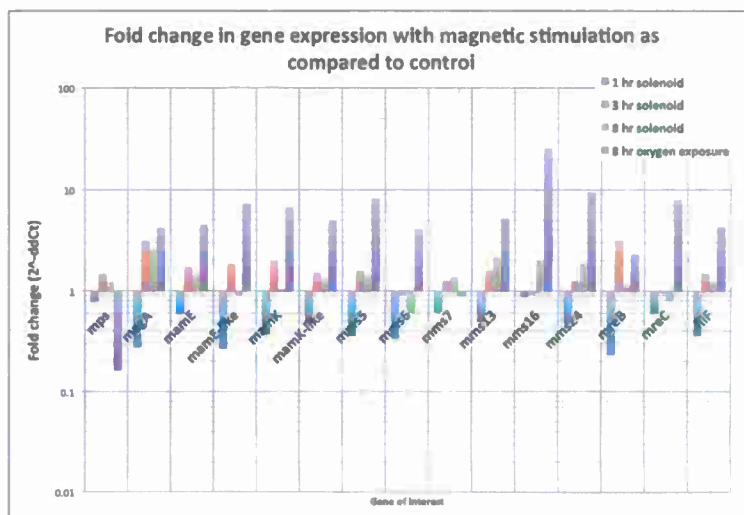


Figure 9. Quantitative RT-PCR gene expression profiles with high magnetic field stimulation (1, 3, 8 hrs) and oxygen exposure (8 hrs).

activation/deactivation, in addition to potentially related cytoskeletal and flagellar regulatory events. After 8 hours of magnetic stimulation, gene expression was relatively unchanged, indicating that the AMB-1 cells may have adapted or equilibrated to the field. Finally, we note that data in this plot (Fig. 9) is shown in relation to housekeeping gene expression, which was relatively constant under all experimental conditions. These findings, we believe, are very promising indications that we can identify genes specifically affected by magnetic stimulation, and provide several significant targets for creating and controlling the genetic response using bacteria. For long-term control, key genes will likely need to be placed under the control of a synthetic memory module, such as a toggle switch.

CONCLUSIONS: We have developed an experimental system to create magnetized structures for examining magnetotactic cell responses to external magnetic fields. We have used our approach to examine the magnetic response of surfaces through a computational analysis approach. We have then experimentally probed the response of *magnetospirillum magnetotacticum* to localized magnetic fields with cell-material interactions. We have also examined the genetic response of the bacteria to applied magnetic fields. Through our studies and based on our results, we conclude that magnetotactic bacteria can be controlled in terms of position and detection of magnetic field gradients. Furthermore, their genetic response for a defined set of associated genes is regulated by applied magnetic fields.

SIGNIFICANCE: Through this approach and our team, which includes researchers from multiple disciplines including engineering, biology, physics, and chemistry, we feel that we have advanced the scientific understanding of these cell-material interactions for future applications. The long term impact potentially include the enabling of future technologies for the detection of defects in naval structures such as ship hulls and also understanding the genetic response of organisms for control using magnetic field stimulation, which we believe are relevant to the US Office of Naval Research.

PATENT INFORMATION (Selected patents to limit the number of report pages):

Wilson, MB, Gonzalez L, Zenkov E, Ruder W, LeDuc PR. Engineering magnetically-inducible gene expression networks in magnetic bacteria (Provisional patent filed)

Kim YT, Messner W, LeDuc PR. Long Term Pressure Regulation using variable resistance and capacitance (Patent Submitted)

Kim YT, Messner W, LeDuc PR. Methods, Apparatuses, and Systems for Three-Dimensional Chemical Pattern Control using Two-Dimensional Modular Microfluidics: Creating 3-D patterns using microfluidics (Patent Submitted)

Gonzalez L, Zenkov E, Messner W, Ruder W, LeDuc PR. Methods, Apparatuses, and Systems for Magnetotactic Bacteria Magnetic Field Control (Provisional Patent Submitted)

AWARD INFORMATION (Selected information to limit the number of report pages):

Board of Directors, Biomedical Engineering Society (www.bmes.org)

The George Tallman Ladd Research Award

Russell Trader Faculty Fellow

Founding Director- Center for the Mechanics and Engineering of Cellular Systems.

Editorial Board, Scientific Reports (Nature Publishing Group)

National Academy of Engineering US-India Frontiers of Engineering, Co-chair of the Bioengineering and Healthcare session

Elected Fellow- Biomedical Engineering Society (1st professor ever at Carnegie Mellon)

Elected Fellow-American Institute for Medical Biological Engineering

Promoted to Full Professor

PUBLICATIONS and ABSTRACTS (for total period of grant):

PUBLICATIONS (Selected from our 28 archival journal publications related to this grant)

Gonzalez, L., Ruder, W. C., Zenkov, E., Messner, W. & LeDuc, P. "Sudden Motility Reversal Indicates Sensing of Magnetic Field Gradients in Magnetotactic Bacteria" Proceedings of the National Academy of Sciences U S A (Second round reviews)

Kim Y, LeDuc PR, Messner WC, Davidson LA. "Mechanochemical actuators of embryonic epithelial contractility" Science (Second round reviews)

Ruder, W.C.*, LeDuc, P.R.* Cells Gain Traction in 3D. Proceedings of the National Academy of Sciences U S A 109, 11060-11-61 (2012).

Ruder, W.C., Chia-Pei, D.H., Edelman, B.D., Schwartz, R.*, & LeDuc, P.R.* Biological Colloid Engineering: Self-Assembly of Dipolar Ferromagnetic Chains in a Functionalized Biogenic Ferrofluid. Appl. Phys. Lett. 101, 063701 (2012).

Tan, C., Lo, J., LeDuc, P.R.*, Cheng, C.-M.* Frontiers of Optofluidics in Synthetic Biology. Lab Chip 12, 3654-3665 (2012).

LeDuc PR*, Messner WC,* Wikswo JP.* 2011. How do controls approaches enter into biology? Annual Reviews of Biomedical Engineering

Kim Y, Joshi SD, Messner WC*, LeDuc PR,* Davidson LA.* 2011. Detection of Dynamic Spatiotemporal Response to Periodic Chemical Stimulation in a Xenopus Embryonic Tissue. PLoS ONE 6(1): e14624.

Lin YW, Bellin RM, Cheng CM, Steward R, Cheng YR, LeDuc PR*, Chen, CC.* 2010. Probing localized neural mechanotransduction through surface-modified elastomeric matrices and electrophysiology Nature Protocols 5 (4): 714-724.

Kim YT, Pekkan K, Messner WC,* LeDuc PR.* 2010. Three-dimensional chemical profile manipulation using two-dimensional autonomous microfluidic control Journal of the American Chemical Society (JACS) 132: 1339-1347.

Bellin, RM*, Kubicek, JD, Frigault, MJ., Kamien, A.J, Steward, R, Barnes, HM, DiGiacomo, MB, Duncan, LJ, Edgerly, CK, Morse, EM, Park, CY, Fredberg, JJ, Cheng, CM, & LeDuc, PR* 2009. Defining the Role of Syndecan-4 in Mechanotransduction using Surface-Modification Approaches Proceedings of the National Academy of Sciences 106: 22102-22107

* Denotes Corresponding Author

ABSTRACTS (Selected from our 55 abstracts related to this grant)

LeDuc, P. R. (2012). KEYNOTE: Planes, Trains, Automobiles . . . and Cells. Techkriti-Indian Institute of Technology-Kanpur.

Wilson, M.E., Gonzalez, L., LeDuc, P., & Ruder, W. C. (2012). Engineering Magnetic Nanomaterial Production in Magnetotactic Bacteria Through Gene Regulation. Proceedings of the American Institute of Chemical Engineers Annual Meeting.

Gonzalez, L., Ruder, W. C., Zenkov, E., Messner, W. C., & LeDuc, P. R. (2012). Sensing of Local, Highly Concentrated Magnetic Field Gradients in Magnetotactic Bacteria Induces Motility Reversal . Proceedings of the ASME 2012 Summer Bioengineering Conference

Zenkov E, Gonzalez L, Ruder WC, LeDuc PR. 2011. Vector construction for genetic manipulation of biogenic nanoparticle producing bacteria. Biomedical Engineering Society, 2011 Oct. 12-15; Hartford, Connecticut.

KEYNOTE: LeDuc PR. 2011. Linking mechanics to biochemistry in molecular and cellular behavior through nano- and micro-technology and biological inspired nanotechnology. Proceedings of the Nanomaterials Symposium "Frontiers in Materials Science for the 21st Century- Applications in Biomedicine"; 2011 May 23-24; Rochester, NY.

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February 8, 2013

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8725 John J Kingman Road Ste 0944

Fort Belvoir, VA 22060-6218

Subject: Submission of Technical Report

Prime Award Number: N00014-09-1-0215

CMU Award Number: 1140180

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Please find enclosed the following document for the above referenced award.

 X Final Technical Report

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Thank you,



Beth Hockenberry

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